Non-volatile Chemical Mutagens in Sediments of the Kanawha River, West Virginia[1]

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ABSTRACT. Sediments from the Kanawha River Basin were examined for the presence of non-volatile chemical mutagens using the *Salmonella*/mammalian microsome assay. Surface sediments were collected at two sites, one upstream and the other downstream from the chemical manufacturing complexes at South Charleston, Institute, and Nitro, WV. Sediment samples were prepared for extraction by milling a portion of wet sediment with anhydrous sodium sulfate. Preliminary chemical fractionation was achieved by extracting milled samples sequentially with Freon-113, methylene chloride, acetone, and methanol. Bacterial mutagenicity tests were performed on each solvent fraction using *Salmonella* tester strains TA98 and TA100, with and without the addition of Aroclor-induced rat liver microsomal (S9) enzymes. Mutagens were recovered from both sampling sites and mutagenic responses were very similar in the two sediment samples. Both sites exhibited more TA98 than TA100 mutagenesis, indicating a greater abundance of frame-shift mutagens as compared to base-substitution type mutagens. The more polar sediment extracts (acetone and methanol) contained more mutagenic residues than did the less polar methylene chloride fractions. No mutagenic activity was recovered in the nonpolar Freon-113 extracts. Methanol extracts from the two sampling sites were different in that the downstream sample contained at least one direct-acting frame-shift mutagen, while the upstream sample was mutagenic only in the presence of S9 enzymes.

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INTRODUCTION

The Kanawha River in West Virginia has been a center of human industrial activity for over two hundred years. Although the river was at one time heavily polluted with municipal and industrial wastes, recent efforts aimed at reducing inputs of biologically hazardous materials have resulted in significant improvements in river water quality (Mason et al. 1971, Mount and Norberg-King 1985). Dissolved oxygen depletion, once common throughout the lower Kanawha Basin, now occurs only rarely; game fish populations have been reestablished and the river is becoming an important local recreational resource (WVDNR 1987a).

Despite these improvements, however, there is evidence of residual chemical contamination of sediments in many parts of the river basin. Studies conducted by the West Virginia Department of Natural Resources (WVDNR) and the U.S. Environmental Protection Agency (USEPA) have demonstrated the presence of polycyclic aromatic hydrocarbons (PAH), heavy metals, and chlorinated pesticides, not only in sediments, but also in tissues of several species of game fish (WVDNR 1986, 1987b).

One approach to the assessment of environmental health risks associated with contaminated sediments and fish tissues in the Kanawha River is to analyze samples for the presence of chemical mutagens. Short-term bioassays for mutagenesis can serve as effective tools for screening large numbers of environmental samples for the presence of biologically hazardous organic chemicals (Schuetzle and Lewtas 1986). The *Salmonella*/mammalian microsome mutagenesis assay has been used to screen synthetic fuels (Guerin et al. 1980), drinking water supplies (Tabor and Loper 1980, Loper and Tabor 1983), cigarette smoke condensates (Rubin et al. 1976), ambient air particulates (Pitts et al. 1982), crude oils (Epler et al. 1978), and diesel particulates (Schuetzle and Lewtas 1986). The *Salmonella* assay has also been used to detect mutagens in sediment samples in the Black River (Ohio) (West et al. 1986), the River Meuse (Belgium) (Van Hoof and Verheyden 1981), and the Nagara River (Japan) (Sato et al. 1983).

The *Salmonella* tester strains lack the ability to synthesize the amino acid histidine and so cannot grow without histidine supplements. Exposure to mutagenic chemicals causes a proportion of the cells to undergo a reverse mutation and regain the ability to synthesize histidine (His+ revertants). Other short-term genotoxicity assays (e.g., unscheduled DNA synthesis, production of micronuclei, sister chromatid exchanges) have been used in screening environmental samples, but these assays are not as well characterized as the *Salmonella* assay (Pesch et al. 1985).

The *Salmonella* assay was used in the present study to survey sedimentary mutagens in the Kanawha River Basin. Sediment samples were collected at two points in the Kanawha River, one near Montgomery, WV, and the other between St. Albans and Nitro, WV. These sites were chosen so as to bracket the industrial and municipal complexes at St. Albans/Nitro, Institute, and South Charleston, WV. The expectation that sediments collected at Montgomery, which is located more than 27 km upriver from the main center of industrial activity, would contain fewer chemical mutagens than those collected at the St. Albans/Nitro site was not met. Sediments from both sampling sites contained a variety of different types of nonvolatile chemical mutagens.

MATERIALS AND METHODS

STUDY AREA. The St. Albans/Nitro sampling site is located at Kanawha River Mile (KRM) 43, approximately 1.9 km upriver from the Donald Legg Memorial (Interstate 64) Bridge (Fig. 1). The Nitro area contains several large chemical manufacturing plants and two abandoned plant sites that are used for chemical waste disposal. The site also lies downriver from large chemical manufacturing complexes at South Charleston and Institute, WV. One sediment sample was obtained at a point approximately 10 m off the left bank in 3 m of water.

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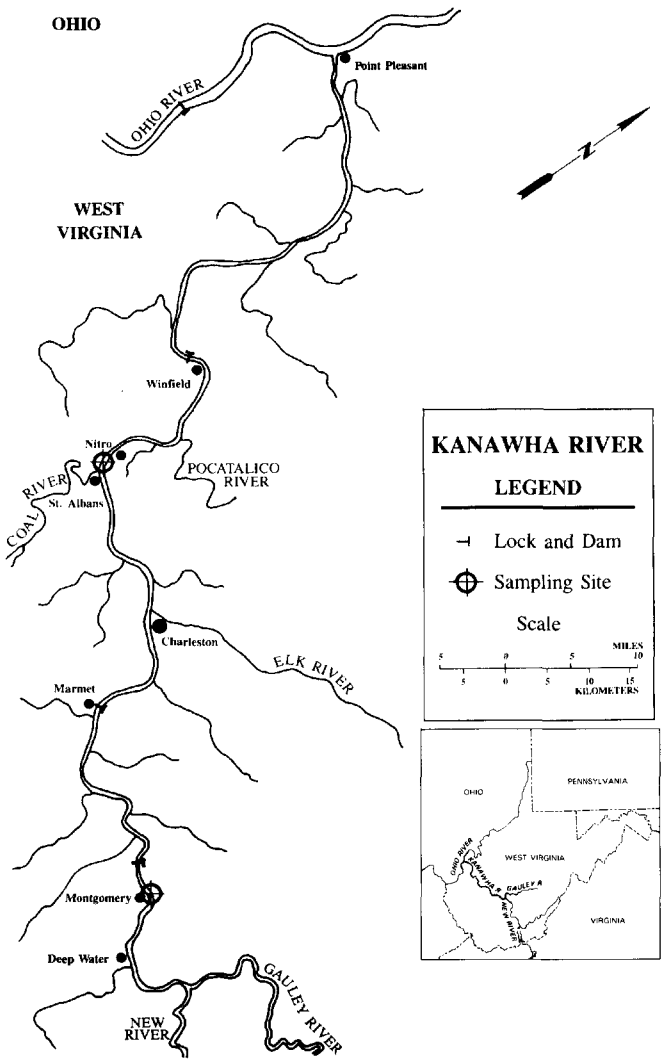

FIGURE 1. Map of the Kanawha River showing locations of sediment sampling sites.

The Montgomery site is located at KRM 86, 0.5 km upriver from the Montgomery Highway Bridge. This area has less industrial activity than the St. Albans/Nitro area, although there are several active coal mines and municipal outfalls nearby. One sediment sample was collected at a point approximately 10 m off the left bank in 3 m of water.

SAMPLING METHOD. Both sediment samples were collected on 17 May 1986. Samples consisting of the top 10 cm of sediment from an area of approximately 0.1 m^2 were collected with an Ekman dredge. Samples were transferred to screw-capped glass jars, covered with aluminum foil to prevent exposure to light, and returned on ice to the laboratory where they were immediately frozen at −70°C.

SEDIMENT EXTRACTION. Sediment extraction was carried out according to the method of Tabor (Tabor et al. 1985, Tabor and Loper 1987). Frozen samples were thawed, allowed to settle overnight at 4°C, and centrifuged at 8000 × g to remove pore water. A portion of each moist sediment pellet was weighed, dried for 24 hr at 105°C, and then reweighed for determination of sediment dry weight.

One hundred gram aliquots of each moist sediment pellet were mixed with 400-500 g of anhydrous sodium sulfate and milled in a 4 L ball mill for approximately 2 hr, until the samples had the consistency of flour. The milling procedure reduces the time required for complete extraction and so inhibits the loss of labile mutagens while minimizing the introduction of extraction artifacts (Tabor and Loper 1987). Milled samples were stored at −70°C prior to extraction.

A 70 g portion of each dried sediment sample was placed in a Soxhlet apparatus and extracted for 2 hr with 250 mL of high performance liquid chromatography (HPLC)-grade 1,1,2-trichloro-1,2,2-trifluoroethane (Freon-113). The samples were allowed to air-dry, and then extracted for 2 hr with 250 mL of HPLC-grade methylene chloride. Additional extractions were carried out in the same fashion with HPLC-grade acetone and methanol, so that each sample produced four 250 mL solutions consisting of one of the four solvents plus all organic residues extracted by that solvent. Each of these solutions was concentrated via rotary evaporation at a temperature of either 30°C for Freon-113 and methylene chloride extracts or 50°C or acetone and methanol extracts. Prior to mutagenicity testing, each extract was further concentrated to a small volume by evaporation under dry nitrogen and then redissolved in 100% dimethyl sulfoxide (DMSO).

MUTAGENICITY TESTING. Mutagenicity testing was carried out as described by Ames (Ames et al. 1975, Ames and Maron 1983) using *Salmonella* tester strains (TA98 and TA100) obtained from his laboratory, with procedural modifications as suggested by de Serres and Shelby (1979) and Venitt et al. (1984). Cultures were checked regularly for induced and spontaneous mutation rates and for the presence of characteristic genetic markers, including biotin and histidine requirements, the "deep rough" (*rfa*) mutation, the presence of plasmid pKM101, and the deletion of the *uvrB* region (Williams and Preston 1983).

Each sediment extract was diluted so as to provide a range of concentrations covering four orders of magnitude. For the St. Albans/Nitro sample, the undiluted solvent extracts contained the organic residues extracted from 437.2 mg (dry weight) of sediment. Thus, a single mutagenicity test at the maximum concentration received the equivalent of 437.2 mg (dry weight) of sediment. Dilutions of the St. Albans/Nitro sample provided the equivalent of 218.6, 109.3, 14.57, 1.46 and 0.15 mg of sediment per test. Corresponding values for the Montgomery sample were 474.8 (undiluted), 237.4, 118.7, 23.74, 2.37 and 0.24 mg of sediment per test.

Solvent extracts from the two sampling sites were tested over the entire four-decade concentration range both in the presence and in the absence of S9, a mixture of cofactors and mutagen-activating enzymes derived from the livers of Aroclor 1254-induced rats. The S9 liver enzymes include monooxygenases that normally render toxic compounds more polar and therefore more easily excreted. However, because oxygenation often makes these compounds more electrophilic, the S9 enzymes can also produce metabolites that readily interact with DNA. Many environmental mutagens, including benzo[a]pyrene and other PAH, require this type of metabolic activation (Phillips 1985). Following the recommendations of Venitt et al. (1985) and Williams and Preston (1983) for preliminary screening of environmental samples, all extracts were tested without activation and also with S9 mixtures containing high (20%) and low (4%) concentrations of commercially-prepared (Organon Teknika, Durham, NC) S9 enzymes.

Each mutagenicity assay included a set of negative controls in which only DMSO was added, and a set of positive controls containing known mutagens (2-aminoanthracene or duanomycin HCl for TA98, 2-aminoanthracene or methyl methane sulfonate for TA100) dissolved in DMSO. All sample dilutions and controls were run in duplicate. Total revertant colonies appearing on the plates after 65 hr were enumerated with a New Brunswick Biotran II automatic colony counter. Colonies appearing on negative control plates were considered to be spontaneous revertants and were used to determine background levels of mutagenicity. Samples were considered mutagenic if they produced at least two times the background number of His+ revertants, and exhibited a linear increase in activity over three or more consecutive extract concentration levels (Williams and Preston 1983).

RESULTS

CONTROLS. Mutagenesis assay spontaneous reversion rates and positive control values (Table 1) were consistent with published standards for the two tester strains. A method control was devised by milling and extracting only the sodium sulfate, and testing these extracts along with the sediment extracts. None of the method control fractions exhibited mutagenic activity.

ST. ALBANS/NITRO SEDIMENTS. Results of the mutagenesis assays were analyzed by dividing the maximum number of His+ revertants obtained from each four-decade range of extract concentrations by the average

TABLE 1

Salmonella/mammalian microsome mutagenesis assay spontaneous reversion rates and positive control values for bacterial strains TA98 and TA100. Values are average His+ revertants ±1 standard deviation. Number of assays is given in parentheses.

Strain		Spontaneous Reversion	Positive Controls		
			MMS* (2 μL)	Duanomycin HCl (5 μg)	2-AA# (2 μg)
TA98	−S9	25 ±1(66)	---	438 ±45(3)	---
TA98	+S9	34 ±1(74)	---	---	779 ±405(8)
TA100	−S9	109 ±3(29)	1233 ±119(6)	---	---
TA100	+S9	98 ±3(32)	---	---	1062 ±387(6)

*Methane methylsulfonate
#2-Aminoanthracene

number of spontaneous (background) revertants for that assay. This resulted in a series of mutagenicity ratios (MR) for each tester strain (Table 2).

Both methylene chloride and acetone extracts of sediments from the St. Albans/Nitro sampling site were mutagenic to strain TA98 (MR ≥2.0; linear concentration-response), but only in the presence of 20% S9. The methanol extract was strongly mutagenic in the absence of S9 (MR = 3.12) and was the only extract capable of inducing mutagenesis without metabolic activation.

TA100 mutagenesis was exhibited only in the acetone extract and only with the addition of 20% S9. The Freon-113 extract produced a mutagenicity ratio of 2.0, but there was no difference in activity when the extract concentration was increased from 218.6 to 437.2 mg sediment per test, so the sample could not be considered mutagenic.

MONTGOMERY SEDIMENTS. The methylene chloride, acetone, and methanol extracts of sediments from the Montgomery sampling site exhibited mutagenicity with strain TA98 and this activity was strongly dependent on S9 enzyme activation (Table 2). The acetone extract with 20% S9 produced over five times the background level of TA98 His+ revertants. The undiluted methanol extract (474.8 mg sediment per test) produced a mutagenicity ratio of 2.11 in the absence of S9, but because this was the only methanol concentration that exceeded the two-times background criterion under these conditions, direct-acting TA98 mutagenesis could not be demonstrated.

TA100 mutagenesis was induced by the acetone extract in the presence of 20% S9; no other Montgomery sediment extracts were mutagenic with TA100.

DISCUSSION

The tester strains used in this study are capable of distinguishing between two major types of chemical mutagenesis. Strain TA98 is sensitive to frame-shift mutagens which, by intercalating between the bases in DNA, may cause a shift in the reading frame when the DNA is transcribed. Most PAH, aromatic amines, aromatic amides, and nitro-aromatic compounds ultimately produce point mutations in this way (Phillips 1985). Strain TA100 is sensitive to base-pair substitution mutagenesis which usually requires incorporation of a nucleotide base analog. Base-pair substitution can also occur via hydroxylation of DNA bases. Alkylating agents such as nitrosoguanidine react chemically with DNA bases and cause both frame-shift and base-substitution mutations (Stanier et al. 1986).

Both types of mutagens were found in the Kanawha River sediment extracts and sediments from the two

TABLE 2

Results of Salmonella/*mammalian microsome mutagenesis assays performed on extracts of surface sediments collected at two sites in the Kanawha River. Mutagenicity is expressed as the ratio of the maximum number of His+ revertants produced by a sample extract divided by the average background response. Each assay was performed with* Salmonella *tester strains TA98 and TA100 at three levels of Aroclor 1254-induced rat liver microsomal enzyme preparation (S9).*

Extraction Solvent	TA98			TA100		
	No S9	4% S9	20% S9	No S9	4% S9	20% S9
ST. ALBANS/NITRO, WV (KRM 43)						
Freon-113	1.13	1.19	1.23	1.16	1.87	2.00
Methylene Chloride	1.44	1.89	2.80*	0.98	1.45	1.64
Acetone	1.30	1.67	2.09*	1.07	1.09	2.17*
Methanol	3.12*	1.11	0.98	0.96	1.07	1.12
MONTGOMERY, WV (KRM 86)						
Freon-113	1.10	1.30	1.31	0.78	1.25	1.00
Methylene Chloride	1.29	2.31*	3.78*	1.29	1.26	1.66
Acetone	1.37	3.63*	5.41*	1.05	1.89	3.73*
Methanol	2.11	2.55*	3.24*	1.19	1.52	1.07

*Indicates linear response to increasing extract concentration.

sampling sites produced very similar patterns of mutagenic responses. Both sites exhibited more TA98 than TA100 mutagenesis. Methylene chloride, acetone and methanol extracts from both sites were mutagenic to TA98, while only the acetone extracts were mutagenic to TA100. This indicates that frame-shift mutagens were more abundant than base-pair substitution mutagens at the two sampling sites. Given the wide variety of sources of frame-shift mutagens, it is not surprising that they would be detected more often. The WVDNR identified 13 different PAH in stream sediments in the St. Albans/Nitro area (WVDNR 1985), at least five of which (benzo[a]pyrene, benzo[g,h,i]perylene, fluoranthene, indeno[1,2,3,-cd]pyrene, and pyrene) are known to be mutagenic to *Salmonella* strain TA98. Functional DNA base analogs would be much rarer and less likely to persist in sediments over long periods of time.

Most of the mutagens detected in this study were promutagens, i.e., mutagens requiring metabolic activation by microsomal (S9) enzymes in order to become genetically active. Many compounds from a variety of different chemical classes are known to undergo metabolic activation to reactive electrophiles. These include PAH, aromatic amines, alkyl and arylnitrosomines, aromatic azo-compounds, aliphatic vinyl-compounds, and many natural products such as mycotoxins and plant allelochemicals. Direct-acting mutagens form DNA adducts without enzymatic activation and include many alkylsulphonic esters, epoxides, aromatic N-oxides, aromatic nitro-compounds, lactones, alkylnitrosoureas and alkylnitrosamides (Venitt and Parry 1984). The only assay that exhibited significant direct-acting mutagenesis in this study was the St. Albans/Nitro methanol extract with strain TA98. This extract was nonmutagenic to TA98 in the presence of S9. The methanol extract of the Montgomery sediment sample produced the opposite response, i.e., positive in the presence of S9 and negative (or at most, weakly positive) in the absence of S9. This indicates the presence of at least two types of methanol-extractable mutagens in the sediments at these locations.

The use of four extraction solvents resulted in a preliminary separation of the residues into four groups based on polarity, with the more polar residues remaining in the methanol and acetone fractions, the less polar residues in the methylene chloride fractions, and the nonpolar residues in the Freon-113 fractions. Most of the mutagenic activity detected in this study was in the acetone and methanol extracts, indicating that the mutagens involved were polar compounds. No mutagenic activity was recovered in the nonpolar Freon-113 fractions. Similar analyses of wastewater treatment plant products have attributed most of the mutagenesis in these materials to nonpolar compounds (Maciorowski et al. 1983, Hopke and Plewa 1983, Tabor et al. 1985). Thus, the chemical nature of sedimentary mutagens in the Kanawha River Basin may be substantially different from that of sewage sludge mutagens.

The recovery of several strong mutagens from the Montgomery sediments was unexpected. The Montgomery site is 70 km upriver from the St. Albans/Nitro site and over 25 km upriver from the main industrial complex at South Charleston, WV. However, the site is by no means free from human impact. The city of Montgomery and several other municipalities discharge treated wastewater at points above and below the sampling site. There is also a large metal finishing plant approximately 5 km upriver from the site.

One potentially important source of chemical mutagens in the upstream sediments is the fine particles of coal that are continuously deposited as a result of natural erosion of coal-bearing strata or because of coal mining and transportation activities on the river. Extracts of these particles could have resulted in mutagenic residues that require S9 enzymes for expression in the *Salmonella* assay (Ribin et al. 1976).

The presence of chemical mutagens in Kanawha River sediment extracts does not, of course, automatically imply that the sediments are environmentally hazardous. Hydrophobic compounds and compounds associated with coal particles may be so tightly bound to the sediment matrix that they remain buried indefinitely in the absence of severe disturbance (Means et al. 1979). However, the possibility of a disturbance leading to dissolution of these materials and subsequent release of mutagens into the water column should not be overlooked. Further analytical work focusing on separation and identification of the compounds responsible for the observed mutagenicity will be required in order to assess the potential environmental impact of sediment-bound mutagens in the Kanawha River.

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